Hypoglycemic Toxins and Enteroviruses as Causes of Outbreaks of Acute Encephalitis-Like Syndrome in Children, Bac Giang Province, Northern Vietnam

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We investigated the cause of seasonal outbreaks of pediatric acute encephalitis-like syndrome associated with litchi harvests (May–July) in northern Vietnam since 2008. Nineteen cerebrospinal fluid samples were positive for human enterovirus B, and 8 blood samples were positive for hypoglycemic toxins present in litchi fruits. Patients who were positive for hypoglycemic toxins had shorter median times between disease onset and admission, more reports of seizures, more reports of hypoglycemia (glucose level <3 mmol/L), lower median numbers of leukocytes in cerebrospinal fluid, and higher median serum levels of alanine aminotransferase and aspartate transaminase than did patients who were positive for enteroviruses. We suggest that children with rapidly progressing acute encephalitis-like syndrome at the time of the litchi harvest have intoxication caused by hypoglycemic toxins, rather than viral encephalitis, as previously suspected. These children should be urgently treated for life-threatening hypoglycemia.

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Acute encephalitis syndrome is clinically characterized by fever, seizures, and altered mental status. This syndrome is a major public health concern in Asia; annually, >133,000 children are hospitalized with this pathology (*1*,*2*). Historically, the main etiology of acute encephalitis syndrome in Asia has been Japanese encephalitis, a vector-borne disease caused by a flavivirus (Japanese encephalitis virus), which causes >25% of cases of this syndrome in Asia. Many other viruses have been shown to cause acute encephalitis syndrome in Asia, such as enteroviruses (e.g., poliovirus, echovirus 9, enterovirus 71), and herpes simplex, measles, varicella zoster, rabies, dengue, Chandipura, and Nipah viruses.

However, for most cases of acute encephalitis syndrome, the specific etiology is unknown (*3*–*5*). Since introduction of Japanese encephalitis vaccine in the Expanded Program on Immunization in Asia (South Korea, China, Bangladesh, and Nepal) in 1997, a major shift has occurred; cases of Japanese encephalitis–attributable acute encephalitis syndrome have decreased, and cases of acute encephalitis syndrome not attributed to Japanese encephalitis have increased in countries with large vaccination coverage (*6*–*10*).

Such a shift has been observed in Vietnam, where the prevalence of Japanese encephalitis for hospitalized patients decreased from 50% in 1996 to 10% in 2009 (T.P. Nga, unpub. data). According to the Ministry of Health, 67% of the 1,800–2,300 cases of acute encephalitis syndrome reported each year occur in northern Vietnam, most

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often in Bac Giang Province (http://moh.gov.vn/Pages/Search.aspx?Key=japanese encephalitis in Vietnam). There has been a clear seasonal pattern of acute encephalitis-like syndrome in this province since 1999, with peaks in summer, particularly in young children (*11*). The symptomatology described by parents as rapid development of fever, headache, and nocturnal seizures explains the local name given to the disease (Ac Mong, meaning nightmare). Local populations had previously suggested a link with litchi cultivation because of observed synchronicity between outbreaks of acute encephalitis-like syndrome and litchi harvests.

A previous investigation of outbreaks of acute encephalitis-like syndrome in Bac Giang Province found that litchi cultivation appeared to be associated with acute encephalitis-like syndrome, but the link at the individual level remained unclear (*11*). Until 2007, results of all virologic investigations on patient samples remained inconclusive. We therefore performed next-generation sequencing (NGS) on cerebrospinal fluid (CSF) samples obtained since 2008 to identify unknown or unforeseen viruses. In addition, because hypoglycin A (HGA) and methylenecyclopropylglycine (MCPG) are suspected to be probable causes of similar outbreaks of acute encephalitis syndrome during litchi harvests in India and Bangladesh (*12–16*), we also tested serum samples for these toxins. These toxins are present in seeds and aril (flesh) of litchis (*17*,*18*) and are known to induce hypoglycemia in animal models (*19*).

Materials and Methods

Study Area and Outbreak Characteristics

Detailed characteristics for this study have been previously reported (*11*). In brief, Bac Giang Province is a rural province that has 1.6 million inhabitants and is located in northern Vietnam. The only hospital is located in Bac Giang City, the capital of the province. Outbreaks of acute encephalitis-like syndrome in Bac Giang Province are unusual and characterized by their specific location, strict seasonality, restricted age group, rapid progression to coma, and a higher case-fatality rate than that for Japanese encephalitis (*11*).

Study Design and Data Collection

We used surveillance data for case-patients with acute encephalitis-like syndrome admitted to the Bac Giang Provincial Hospital during 2008–2011. The case definition used for this syndrome was fever (temperature ≥37°5C reported by parents or at hospital admission), altered mental status or seizures, and no bacterial meningitis. We collected data from the Bac Giang Preventive Medicine Centre and the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). Patients ≤15 years of age, those who had onset of acute encephalitis-like syndrome during May 1–August 31, and those who had negative results for Japanese encephalitis virus IgM in CSF or were immunized against Japanese encephalitis virus were included in the study.

CSF and Blood Samples

For each seasonal outbreak that occurred during 2008–2011, blood samples were obtained from patients with acute encephalitis syndrome at admission to Bac Giang Provincial Hospital for standard biochemical and hematologic analysis. CSF samples from patients were also collected by physicians. Samples were cryopreserved in liquid nitrogen for transportation and stored at –80°C. Because most patients were young children, only small volumes (20 μL–300 μL) of CSF were available for most samples. Because of age of patients and local cultural practices, no brain biopsies or necropsies were performed for children who died of acute encephalitis-like syndrome.

Virologic Analyses

We conducted virus isolation in RD, Vero E6, or C6/36 monolayer cells and PCR for known viruses (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-1004-Techapp1.pdf). In addition, we also performed random NGS for 16 selected CSF samples that matched the case definition and were collected during 2008. We performed specific PCRs for contigs identified by NGS to confirm results, which also enabled comprehensive phylogenetic analysis by using other methods (online Technical Appendix). Primers (online Technical Appendix Table 1) were used to test CSF samples obtained from patients who had available clinical data (3 in 2008, 3 in 2009, 14 in 2010, and 21 in 2011).

Toxicologic Analysis

We tested 20 blood samples obtained during 2010–2011 (4 in 2010 and 16 in 2011, the only ones available from patients who had clinical data at the time when the hypoglycemic toxins hypothesis was proposed) for HGA and its metabolites and metabolites of MCPG by using a modified analytical method that has been reported (*20*,*21*). This method is based on ultra–high-performance liquid chromatography/tandem mass spectrometry.

Because these toxins can cause hypoglycemia by blocking the fatty acid β-oxidation pathway, we also measured concentrations of glycine and carnitine conjugates of short-to-medium chain length fatty acids in the same samples by using the same method. In addition, we quantified a spectrum of carnitine esters of 24 saturated and unsaturated fatty acids ranging from short to long chain molecules (C2–C18), including hydroxyl and dicarboxylic acids, by using tandem mass spectrometry without preceding chromatographic separation (*22*,*23*) (online Technical Appendix).

Statistical Analyses

We compared proportions of continuous variables across groups by using the Fisher exact test and distributions of continuous variables across groups by using the Mann-Whitney U test (R version 3.2.3; R Foundation for Statistical Computing, Vienna, Austria). We performed principal component analysis for age, number of days between symptoms and disease onset, glycemia at admission, number of leukocytes in CSF, and serum levels of liver enzymes to identify grouping of characteristics that might help differentiate between infectious and toxic causes of acute encephalitis-like syndrome. We conducted principal component analysis by using Qlucore Omics Explorer software (Qlucore, Lund, Sweden).

Ethics

Informed consent was obtained by physicians from parents of hospitalized children before sampling was conducted. The study protocol was reviewed and approved by institutional review boards at the National Institute of Hygiene and Epidemiology and the Institut Pasteur (Paris, France).

Results

A total of 185 children met the inclusion criteria over the study period (2008–2011). Median age was 5 years (interquartile range 2–8 years), and the sex ratio (male:female) was 1.4:1. The annual number of cases was higher in 2008 (70) and 2011 (61) than in 2009 (27) and 2010 (27) (Figure 1). Because of logistical constraints, CSF and blood samples were available for only 61 of the 185 children, of which 58 also had detailed clinical data. Therefore, these 58 patients represent the study population analyzed (Figure 1), including 10 patients from a previous study (*11*).

Virologic Analyses

NGS analysis of a pool of 16 CSF samples from the 2008 outbreak provided 116,615 nonhuman contigs from 61,291,294 nonduplicated reads with an average length of 70 nt. Among these contigs, 57 with an average length of 292 nt (range 103 nt–815 nt) matched the human enterovirus B species. Fourteen contigs were assigned to the human echovirus 30 species strain Zhejiang/17/03/CSF (GenInfo Identifier DQ246620) as best hit, with nucleotide identities ranging from 83% to 98%. The second most common reference strain matched (7 contigs assigned, with best-hit ranging from 78% to 88%) was human echovirus 33 strain Toluca-3 (GenInfo Identifier 34485451). Distinct contigs mapped at same genomic locations of these 2 reference genomes and suggested that the pool of samples presumably contained ≥4 different virus strains. PCRs using primers designed for these contigs confirmed their sequences, identified distinct viruses in the pool, and identified patients from which the sequences had been isolated.

We then conducted individual NGS on 4 selected CSF samples from the pool and acquired virus genome sequences after amplification by using specifically designed PCRs. This sequencing identified 4 distinct enterovirus genomes (120486, 120492, 120488, and 120495); the first 2 genomes were closely related (online Technical Appendix Figures 1–3). Prevalence of enterovirus infection was screened by PCR for CSF samples from patients with clinical data available and showed highly variable results (from 13/19 in 2008 to 4/21 in 2011) (Figure 2). Virus isolations were attempted for 10 CSF samples per annual outbreak; all showed negative results.

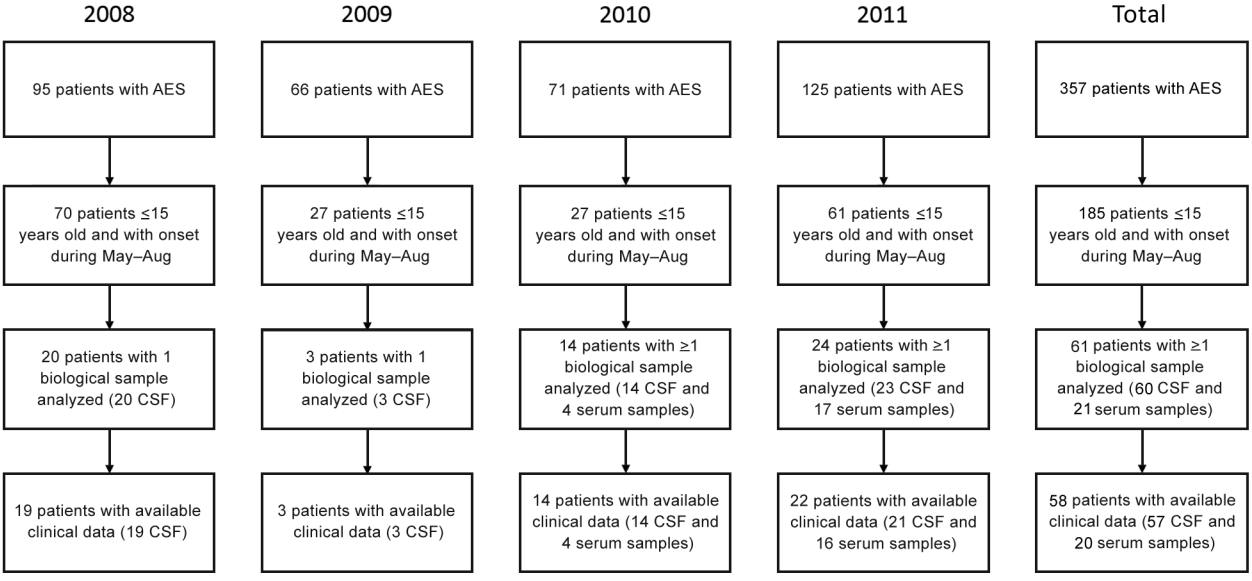

Figure 1. Inclusion of patients in study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in children, Bac Giang Province, northern Vietnam, 2008–2011. AES, acute encephalitis syndrome; CSF, cerebrospinal fluid.

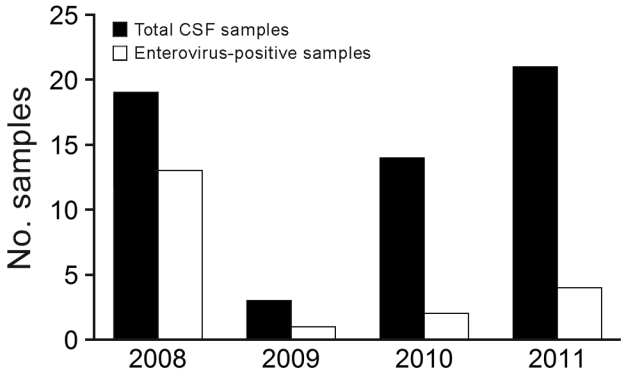

Figure 2. PCR-based prevalence of enterovirus infections per year in study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in samples (n = 57) from children, Bac Giang Province, northern Vietnam, 2008–2011. CSF, cerebrospinal fluid.

Toxicologic Analysis

Although enteroviruses accounted for most (68%) cases in 2008, these viruses accounted for <20% of cases in 2009–2011. Moreover, identification of multiple and distinct enterovirus strains did not correlate with the model of an epidemic diffusion that would otherwise explain seasonal outbreaks. Therefore, we were interested in exploring alternative explanations, including 2 candidate toxins, MCPG and HGA (*13*–*15*,*17*,*18*,*24*). Twenty blood samples (4 from 2010 and 16 from 2011), which were obtained near the time of onset of symptoms, were available for this analysis. After serum analysis (Figure 3; online Technical Appendix Table 2), we categorized children into 2 groups: 9 had high (>100 nmol/L) serum values of HGA and 11 had serum values of HGA below the lower limit of quantification (10 nmol/L), including 10 below the lower limit of detection (1 nmol/L).

All patients with high levels of HGA had quantifiable concentrations of methylenecyclopropylformyl (MCPF) carnitine, a metabolite of MCPG, and 6 of the 9 patients had detectable MCPF glycine, also derived from MCPG, although below the lower limit of quantification. Methylenecyclopropylacetyl conjugates, derived from HGA, were present in all of these samples, but concentrations did not reach quantifiable levels in all cases. The β-oxidation of fatty acids (online Technical Appendix Table 2) was shown to be inhibited in all patients with high serum levels of HGA. As a result, concentrations of glycine and carnitine conjugates of fatty acids of short to long chain length were increased. Increased concentrations were also detected for even and odd chain length acyl compounds and unsaturated compounds in the same samples, which demonstrated complete inhibition of β-oxidation of fatty acids. Of the 9 children who had high levels of HGA/MCPG, 8 had a CSF sample tested: 1 child was positive for enteroviruses, and 7 children were negative. Of the 11 children with low levels of HGA/MCPG, 4 children were positive for enteroviruses, and 7 children were negative.

Relationship between Epidemiologic, Clinical, and Biological Findings and Etiologies

Of the 9 children with high levels of HGA/MCPG, 8 were hospitalized in July 2011 and came from the same eastern district (Luc Ngan), a district known to have the highest levels of litchi production in Bac Giang Province (50% of province production) and in which the harvest occurs each year during June–July (Figure 4). Toxin-negative samples and enterovirus-positive samples were predominantly identified in the western part of the province.

On the basis of results of virologic and toxicologic analyses, we compared clinical and biologic characteristics among 4 patient groups: 1) the 19 children who were positive for enteroviruses and who had either low levels of HGA/MCPG (n = 4) or no blood sample tested for toxins (n = 15); 2) the 8 children with high blood levels of HGA/MCPG and who were negative for enteroviruses (n = 7) or not tested for enteroviruses (n = 1); 3) the 7 children who were negative for enteroviruses and toxins; and 4) the 23 children who were negative for enteroviruses and who were not tested for toxins (Table; online Technical Appendix Table 3). One child was positive for enteroviruses and toxins and was therefore not included in statistical comparisons. All children included in the study had fever either before admission (reported by parents) or at admission, except for 1 child who had high levels of toxins, but no reported fever, who was included in the study because of severity of the neurologic condition of the child (repeated seizures and coma).

Children with high blood levels of HGA/MCPG had shorter median time between disease onset and admission (0 days vs. 2 days; p = 0.008), more seizures (88% vs. 28%; p = 0.009), more hypoglycemia (glucose level <3 mmol/L) (71% vs. 0%; p = 0.001), lower median numbers of leukocytes in CSF (3 cells/mm^3 vs. 50 cells/mm^3; p = 0.001), and higher median serum levels of alanine aminotransferase (48 IU/L vs. 24 IU/L; p = 0.04) and aspartate aminotransferase (68 IU/L vs. 28 IU/L; p = 0.01) than patients infected with enteroviruses. Two (25%) of 8 children who were positive for toxins died, whereas only 1 (5.3%) of the 19 children with enterovirus encephalitis died, but this difference was not significant (p>0.05).

Principal component analysis showed that children with high levels of HGA/MCPG clustered differently in the projection space (online Technical Appendix Figure 4) than children with evidence of infection with enteroviruses (online Technical Appendix Table 4). Furthermore, children not infected with enteroviruses for whom HGA/MCPG showed negative results or was not tested had

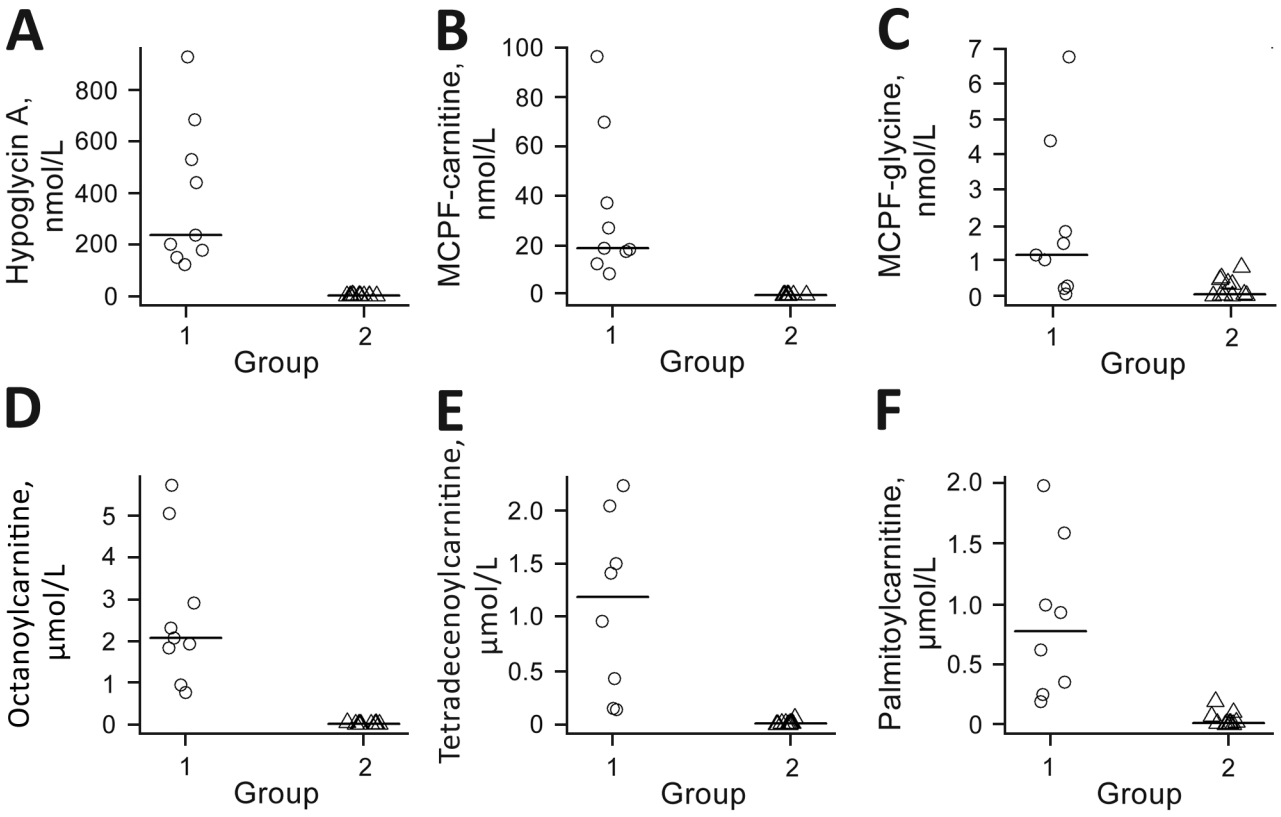

Figure 3. Serum concentrations of toxins and fatty acids in children with encephalitis-like syndrome, Bac Giang Province, northern Vietnam, 2008–2011. Children were grouped by high (group 1, n = 9 [circles]) and low (group 2, n = 11 [triangles]) serum concentrations of toxins. A) Hypoglycin A; B) MCPF-carnitine (methylenecyclopropylglycine metabolite); C) MCPF-glycine (methylenecyclopropylglycine metabolite); D) octanoylcarnitine (medium-chain fatty acid); E) tetradecenoylcarnitine (long-chain fatty acid in the form of acylcarnitine); and F) palmitoylcarnitine (long-chain fatty acid in the form of acylcarnitine). Horizontal lines indicate medians. MCPF, methylenecyclopropylformyl.

profiles more similar to children infected with enteroviruses. In particular, these children had glycemia (glucose level >3 mol/L), and leukocyte counts in CSF were slightly increased (online Technical Appendix Table 3).

Discussion

This study suggests that acute encephalitis-like syndrome previously associated spatially and temporally with litchi harvests (*11*) was caused by intoxication, rather than viral encephalitis, as initially suspected. In this context of recurrent acute encephalitis-like syndrome outbreaks since 1999, no consistent viral etiology had been identified by using standard laboratory diagnostic techniques. Because of the high number of viruses known to be associated with encephalitis (*3*,*5*,*25*), we used NGS to analyze samples of patients after the annual outbreak in 2008. This hypothesis-free technique identified human enterovirus B serotypes that were further confirmed by PCR. Other pathogens were not identified.

Despite the link shown in this study between several acute encephalitis-like syndrome cases and enteroviruses, the frequency of enterovirus infection among clinical cases was highly variable from year to year (Figure 2). Therefore, we tested serum samples of a subset of enterovirus-negative and enterovirus-positive cases for HGA and MCPG metabolites. Of 20 children tested, 9 (45%) were positive for HGA and MCPG metabolites and 8 showed inhibition of the β-oxidation catabolic process. Eight of these 9 case-patients came from the same district, which is known for having the highest litchi production in the province. These case-patients were hospitalized in July 2011, at the time of the litchi harvest in the district. These 8 case-patients appeared to have distinct characteristics in comparison with those who had enterovirus acute encephalitis syndrome, including younger age, more rapid progression, higher frequency of seizures, severe hypoglycemia, lack of increased numbers of leukocytes in CSF, and moderate increases in levels of liver enzymes.

The clinical and biochemical presentation of these case-patients clearly matches that of case-patients reported during outbreaks linked to litchi harvests in India and Bangladesh (*12*,*15*,*16*) and of case-patients with Jamaican

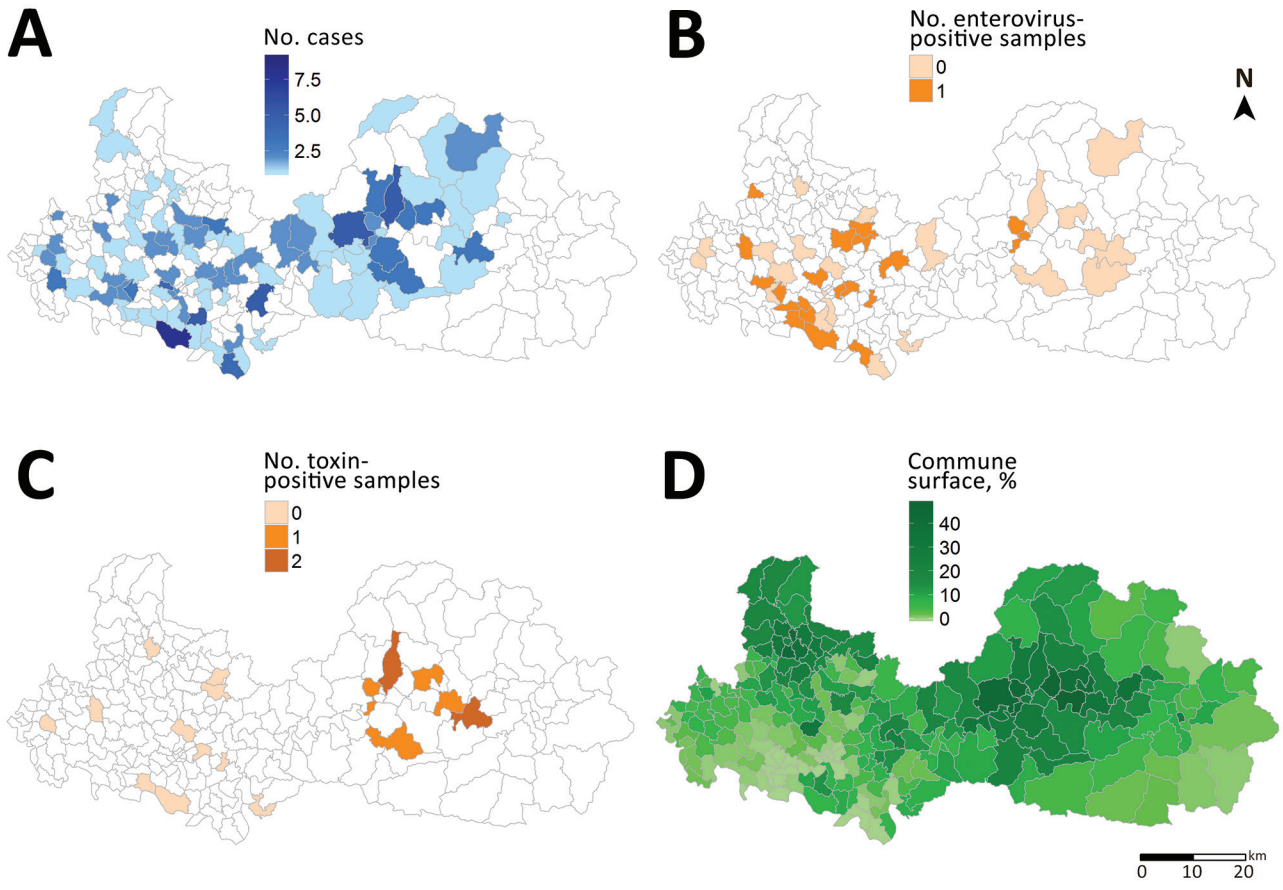

Figure 4. Geographic distribution of acute encephalitis-like syndrome in children, samples, and litchi cultivation at the commune level in Bac Giang Province, northern Vietnam, 2008–2011. A) No. cases of acute encephalitis-like syndrome meeting the inclusion criteria (n = 185); B) no. enterovirus-positive samples among all cerebrospinal fluid samples analyzed (n = 57); C) no. toxin-positive samples among all blood samples analyzed (n = 20); D) percentage of commune surfaces devoted to litchi cultivation.

vomiting sickness (*24*). Investigation of an outbreak in Muzaffarpur, India, recently concluded that intoxication with HGA and MCPG was responsible for the outbreak, a finding that is consistent with our results (*26*), which also identified HGA/MCPG in young patients with hypoglycemic encephalopathy. Our study also provides a direct comparison of clinical and biologic profiles of acute encephalitis-like syndrome related to enterovirus infection versus intoxication with HGA/MCPG. Thus, we provide useful information that can be used to guide clinical decision making, particularly the need for glycemia testing for management of patients with acute encephalitis-like syndrome.

Our study was limited by comparison of children subjected to different tests at different times (only blood samples obtained during 2010 and 2011 were available for testing of toxins, whereas testing for enterovirus was available throughout the study) and by having used fever as an inclusion criteria. In the study by Shrivastava et al. in India, in which this inclusion criterion was not used, 61% of children with litchi intoxication were afebrile (*26*). As a result of using fever as an inclusion criteria, our study might have missed several children with HGA/MCPG intoxication. Apart from 1 patient who had enterovirus infection and high serum concentrations of hypoglycemic toxins, patients with enterovirus infections did not have higher toxin levels than patients without enterovirus infections, suggesting that subtoxic concentrations of HGA/MCPG were not associated with increased risk for enterovirus infection. However, studies with larger numbers of patients are needed to rule out this hypothesis.

We also tried to elucidate the link between these 2 etiologies (enteroviruses and hypoglycemic toxins) and litchi harvesting in northern Vietnam. For enteroviruses, it is likely that temperature and humidity conditions required for enterovirus circulation match those of litchi maturation and harvest. For intoxication with HGA and MCPG, the link is more obvious because toxins have been previously identified in the litchi aril and litchi seeds (*17*,*18*). In our study, cases of intoxication clustered geographically in areas of large production of litchi. Levels of hypoglycemic

Table. Characteristics of 27 children hospitalized with acute encephalitis syndrome who were positive for enteroviruses or toxins, northern Vietnam, 2008–2011*

Characteristic	Enterovirus positive and toxin negative (n = 4) or not tested (n = 15)		Toxin positive and enterovirus negative (n = 7) or not tested (n = 1)		p value
	No. with data	No. (%) or median (IQR)	No. with data	No. (%) or median (IQR)	
Sex	19		8		0.80
F	NA	6 (32)	NA	3 (38)	NA
M	NA	13 (68)	NA	5 (62)	NA
Age, y	19		8		0.47
<2	NA	4 (21)	NA	3 (38)	NA
2–4	NA	5 (26)	NA	3 (38)	NA
5–9	NA	7 (37)	NA	1 (12)	NA
10–15	NA	3 (16)	NA	1 (12)	NA
Symptoms/signs before and at admission					
Temperature at admission, °C	18	38.0 (37.6–38.5)	7	37.5 (37.4–37.8)	0.14
Fever before admission	18	18 (100)	7	5 (71)	0.07
Headache	19	11 (58)	6	3 (50)	1.0
Seizures	18	5 (28)	8	7 (88)	0.009
Coma	14	4 (29)	7	4 (57)	0.35
Meningeal symptoms	18	12 (67)	7	4 (57)	0.67
Limb paralysis	18	1 (6)	5	0	1.0
Vomiting	18	14 (78)	7	6 (86)	1.0
Diarrhea	14	3 (21)	6	0	0.52
Days from disease onset to admission	19	2.0 (0.5–2.5)	8	0.0 (0.0–0.2)	0.008
Blood sample					
Leukocytes, × 10^9 cells/L	18	10.5 (7.5–15.8)	8	19.5 (18.4–29.9)	0.004
Platelets/μL	12	254 (197–306)	7	340 (274–487)	0.20
Hemoglobin, g/L	7	114 (108–118)	6	116 (92–122)	0.77
Glucose, mmol/L	14	4.5 (3.9–5.0)	7	2.0 (1.6–3.8)	0.67
Glucose <3 mmol/L	14	0	7	5 (71)	0.001
Cerebrospinal fluid sample					
Leukocytes/mm^3	12	50 (6–100)	6	3 (1–3)	0.001
Lymphocytes/mm^3	8	80 (73–80)	1	45 (45–45)	0.31
Protein level >0.5 g/L	15	4 (27)	6	0	0.28
Transparent appearance of CSF	15	15 (100)	7	7 (100)	1.0
Liver enzymes at or after admission, IU/L					
Alanine aminotransferase	8	24 (12–33)	8	48 (37–56)	0.04
Aspartate aminotransferase	8	28 (20–46)	8	68 (62–79)	0.01

*CSF, cerebrospinal fluid; IQR, interquartile range; NA, not applicable.

amino acids in the litchis are not known. Results from 2 studies suggest that MCPG concentration is highest in the seeds, followed by arils of semiripe litchis and then ripe litchis (*17*,*26*). Further investigations should compare levels of toxins across cultivars and soil, climate, and harvest conditions, as recommended by Spencer et al. (*27*). To further investigate a causal link between HGA/MCPG levels and acute encephalitis-like syndrome, healthy children exposed to the same litchi intake would need to be tested. Nevertheless, the evidence of inhibited β-oxidation of fatty acids in all HGA/MCPG–positive patients in this study is a convincing demonstration that intoxication was a key driver of symptoms in these patients.

Intoxication with HGA/MCPG is attributed mainly to a hypoglycemic encephalopathy, secondary to inhibition of β-oxidation and an inability to produce glucose from fatty acids. This metabolic process usually takes hours, which might explain why most children have initial symptoms during the second half of the night. Shrivastava et al. reported that children who had no evening meal were at higher risk for developing hypoglycemic encephalopathy (*26*). Young children, and even more so undernourished children, have limited glycogen stores, which increases their vulnerability to the effects of intoxication with HGA/MCPG on metabolism (*13*,*15*). Concentrations of glycine and carnitine conjugates measured in serum samples might appear rather low. These conjugates would be better measured in urine samples (*28*). However, such samples were not available in this study.

In conclusion, this study has shown that within a context of largely viral encephalitis, particularly encephalitis caused by enteroviruses, acute hypoglycemic encephalopathy developed in some children in Vietnam during the litchi harvest, possibly after absorption of a toxin present in the aril of litchi fruits. Local populations should be sensitized to the risks associated with young children eating litchis. Also, for children coming to healthcare facilities because of acute encephalitis-like syndrome during the litchi harvest season, measurement of blood glucose concentrations and immediate infusion with dextrose for those children with hypoglycemia

should be critical elements of clinical management. Use of these elements will likely increase patient survival.

Acknowledgments

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Hypoglycemic Toxins and Enteroviruses as Causes of Acute Encephalitis-Like Syndrome in Children, Bac Giang Province, Northern Vietnam

Technical Appendix

Materials and Methods

Virus Isolation

Whenever sufficient material was available, we inoculated cerebrospinal fluid (CSF) samples onto RD, Vero E6, or C6/36 monolayers. Cell cultures were checked daily for eventual cytopathic effects and harvested 7 days after inoculation. Viral replication was tested by using reverse transcription PCR (RT-PCR).

Purification of Nucleic Acids and Random Amplification

Nucleic acids were purified from 150 µL of CSF by using the NucleoSpinDxVirus Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany), according to the manufacturer's instructions. Purified nucleic acids were eluted in 60 µL of buffer. Eight microliters of purified nucleic acids (containing RNA) was reverse transcribed by using the First-Strand Synthesis Superscript III Kit (Invitrogen, Carlsbad, CA, USA). Complementary DNA and DNA were then amplified by using the Whole Transcriptome Amplification Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Resulting products were used undiluted or diluted up to 1,000 times in nuclease-free ultrapure water before use in preparation of libraries for high-throughput sequencing (HTS) or as a template in specific PCRs (*1*–*5*) (Technical Appendix Table 1).

Virus Detection by RT-PCR

CSF samples were screened by using RT-PCR for viruses already associated with acute encephalitis syndrome. In brief, we tested for herpes simplex virus, measles virus, varicella zoster

virus, enterovirus, dengue virus, and Nipah virus. Alternatively, because of limited amounts of samples, PCR were performed on whole transcriptome amplification products. Data obtained from HTS of pooled samples from the outbreak in 2008 enabled us to design specific RT-PCRs for screening future outbreaks (2008–2011). Conditions for amplification using in-house primers (Technical Appendix Table 1) were at 95°C for 2 min; followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final extension step at 72°C for 5 min. These in-house PCRs were complemented by the use of a PCR specific for conserved regions of enterovirus genomes (*1*–*5*) (Technical Appendix Table 1) and the Argene Enterovirus Detection Kit (bioMérieux, Marcy l'Etoile, France).

Preparation for HTS

The strategy was to explore the metagenome of 16 CSF samples from patients matching the case definition for acute encephalitis syndrome during the outbreak in 2008. A total of 0.2 μg of nucleic acid from each amplified product was pooled and analyzed by using HTS. The 400-nt library was prepared from the pool by using the TruSeq DNA Kit (Illumina, San Diego, CA, USA) and paired-end sequenced by using an HiSeq 2000 Apparatus (Illumina). PCRs designed for contigs sequenced from first-round HTS data enabled selection of 4 CSFs that were individually deep sequenced by using the same method to obtain larger genomic sequences.

HTS and Raw Data Processing

Sequencing was performed on a half-lane of a HiSeq 2000 sequencer for each CSF sample. Raw data (70Mio reads for each CSF) processing consisted of 3 successive filtering steps: duplicated reads removal; quality trimming according to the Phred score quality (minimum of 15 at 3′ and 5′, minimal length after trimming set to 30 nt); and host removal (Soap2 mapping against hg18) (*6*). Remaining reads (31Mio reads) were assembled by using CLC de novo assembly software (CLCBio; QIAGEN). Contigs were aligned against whole nucleic acid and protein databases (https://www.ncbi.nlm.nih.gov/) by using Smith-Waterman algorithm (default parameters but with mismatch penalty set to –1). Taxonomic assignation was performed by using a best-hit strategy; the first alignment was considered and the taxonomic identifier was selected.

HTS Alignment on a Reference Strategy

The best-hit strategy enabled us to select the closest genome reference for a specific taxonomic identifier. All generated contigs were aligned to these references by using a local Smith-Waterman alignment. Relevant alignments have been selected to design PCR primers for Sanger sequencing. In

addition, a mapping strategy (Soap2 aligner) was used to produce coverage and depth of sequencing statistics for each complete genome.

PCR and Sanger Sequencing

PCR products were obtained and sequenced by using in-house–designed primer pairs and pan-enterovirus primer sets (*1*,*3*–*5*). PCR amplifications were performed by using Taq DNA polymerase (Invitrogen). Conditions for amplification by using in-house primers are detailed above. Nucleotide sequences were obtained by using Big Dye Version 1.1 chemical analysis on an ABI 3730XL Apparatus (Applied Biosystems, Foster City, CA, USA).

Genome Analysis and Phylogenetics

Presence of open reading frames in sequenced genomes was investigated by using CLC 4 Main Workbench software version 6.8.4 (QIAGEN). Closest similarities with known enteroviruses were determined by using the BLAST algorithm on viral protein 1 (VP1) and other major open reading frames (*7*). The position and likelihood of recombinations were evaluated by a number of recombination detection algorithms (RDP, geneconv, Bootscan, Maxchi, Chimera, SIScan) by using the RDP3 package (*8*). Using the same complete genomes database, we inferred a VP1-based phylogeny of enteroviruses by using sequences detected in this study and those of 82 serotypes of Human Enterovirus B available on public databases. The following sequences were used: AF326751, HM777023, X77708, AY302542, AY302540, AY302549, AY302539, AY302551, AY302550, EF174469, AF081485, AY843302, AY843297, AY843300, JQ041368, AF105342, DQ480420, X05690, AY843299, EF066392, AY896767, AY302553, E6U16283, AF268065, AY429470, AF230973, AF114383, JN695051, AY896761, JQ729993, AF524866, X84981, CXA9CG, AY302547, EF371880, AF465516, AY896763, GU109481, NC001472, CXA1G, AY843301, AY843298, AY302557, AY302543, CXA3G, AF241359, AY302552, AY302546, AY302556, AY302541, AY302555, AY302544, AY302560, AY302548, AF465518, AY302554, HM775882, AF083069, AY843307, AY896765, AY843304, AJ577594, AY556070, AY556057, AY896764, NC009887, NC013115, AY843303, NC013114, AY843305, AY843306, AY843308, DQ902712, HM777023, AM236984, FJ868349, AM492410, HQ897643, and FJ525936.

Sequences were first aligned by using Muscle software (*9*). The CLC main workbench 6.8.4 and Mega5 software were also used to manipulate the sequences and alignments (*10*). The Bayesian Information Criterion, the corrected Akaike Information Criterion scores, and maximum-likelihood

values were calculated by using jModeltest software (*11*). A Bayesian model was set up and tested by using BEAST (*12*).

According to corrected Akaike Information Criterion estimations, the most appropriate pattern of substitutions was as previously used and was complemented by a discrete gamma distribution of evolutionary rates among sites, assuming that certain sites remained invariable. An uncorrelated lognormal distribution with a default uniform prior ranging from 0 to 1 was used to model the rate of evolution. Several coalescent based models were tested as tree priors. The Markov chain Monte Carlo was set to 10,000,000 states, and 10,000 trees were sampled to obtain an adequate posterior effective sample size >200.

Identification of Recombinants

Virus genomes were included in recombination detection tests and molecular typing approaches. Phylogenetic analysis showed clustering of VP1 sequences from each sample with respective serotypes of human enterovirus B previously identified by the BLAST analysis (Technical Appendix Figure 1). Other parts of the genome, such as genes 2 (A, B, C) and 3 (A, C, D), clustered differently than VP1 and had robust statistical support, indicating that enteroviruses from 2008 were potential recombinants (Technical Appendix Figures 2, 3). Specific analyses of recombination statistically supported several putative recombination breakpoints (Technical Appendix Figure 2).

Interpretation

Among human enterovirus B serotypes known to be associated with encephalitis (*13*), E6, E9, E16, E30, and E3 were reported in this study. Serotypes E6, E30, and E16 have been reported as a cause of meningitis and, more recently, human enterovirus B (CVA9, CVB1–5, E3–7, E9, E11, E13, E14, E16–19, E24, E25, E27, E30, and E33) have been associated with encephalitis in China (*14*,*15*). The serotypes detected in our study are present in recombinant viruses (Technical Appendix Figures 2, 3). The severe clinical presentation observed with recombinant serotypes circulating during outbreaks might be related to a putative role of recombination in pathogenesis, as proposed by Lukashev et al. for E30 (*16*). The recombinants described in our study might result from intense cocirculation of diverse enteroviruses in an ecologic context that might favor emergence of new variants of neurovirulent human enterovirus B.

Toxicologic Analysis

Blood samples were tested for hypoglycin A (HGA), carnitine and glycine conjugates of methylenecyclopropylacetyl (metabolite of HGA) and methylenecyclopropylformyl (metabolite of

methylenecyclopropylglycine [MCPG]), and short-to-medium length chain fatty acids by using a modification of a reported analytical method (*17*,*18*). Sample volume was increased and dilution of extracts was reduced to increase sensitivity. In brief, a methanolic internal standard solution (300 μL) was added to 25 μL of serum or urine for extraction, and the mixture was vortexed for 20 s and centrifuged for 10 min at a relative centrifugal force (RCF) of 17,000. From the clear supernatant, 250 μL was removed and dried in a microtiter plate at 65°C for ≈30 min under a gentle stream of nitrogen. The residue was treated with 50 μL of 3N butanol-HCl for 15 min at 65°C and dried again at 65°C under nitrogen. The dry material was dissolved in in 70 μL of methanol:water (80:20 vol/vol) and further diluted 1:2 with water. From this solution 90 μL was transferred to a 384 microtiter plate, centrifuged at an RCF of 17,000 to sediment any particles, and then used for ultraperformance liquid chromatography–tandem mass spectrometry. From this solution, 5 μL was injected onto an ACQUITY UPLC BEH C18 1.7 μm, 2.1 × 50 mm column (Waters, Eschborn, Germany) for gradient chromatography. Tandem mass spectrometric analysis was performed with single-point calibration on a Xevo TQ-MS UPLC-MS/MS System (Waters). The lower limit of detection for all compounds was ≈1 nmol/L, and the lower limit of quantification was 10 nmol/L.

In addition, a spectrum of 24 carnitine esters of saturated and unsaturated fatty acids ranging from short-chain to long-chain molecules (C2–C18), including hydroxy and dicarboxylic acids, was quantified by using tandem mass spectrometry without preceding chromatographic separation, according to standard methods used in newborn screening for inborn errors of metabolism (*19*,*20*). Serum was diluted 1:5 with water, and 17.5 μL of this solution were extracted with 100 μL of methanol containing 13 deuterized internal standards. After centrifugation at an RCF of 17,000, the supernatant was transferred to a microtitration plate and dried under a gentle stream of nitrogen at 65°C. Butylation of the carnitine esters of fatty acids and of free carnitine was performed by adding 50 μL of HCl-butanol and incubating for 15 min at 65°C. After drying, the residue was dissolved in 200 μL of methanol/water (80:20 vol/vol). From this solution, 20 μL was injected directly onto the electrospray ionization interface of a tandem mass spectrometer (Quattro Micro, Waters, Eschborn, Germany). Concentrations of acyl carnitines were deduced by comparing peak height to those of internal standards.

Principal Component Analysis

Methods

To study clinical and biologic profiles of the 58 patients, principal component analysis (PCA) was performed for age, temperature at admission, number of days between disease onset and symptoms, glycemia at admission, number of leukocytes in CSF, and blood levels of liver enzymes (aspartate aminotransferase and alanine aminotransferase). We determined which groups of patients were similar or different and characterized these groups by variables or groups of variables. Variables were normalized before performing PCA. We excluded from the analysis 1 person who had an excessive contribution to the first principal component (exceeding its weight), which could have caused unreliable results.

Results

We retained the first 3 principal components, which had eigenvalues >1, indicating that they accounted for more variance than accounted by 1 of the original variables. Together, they accounted for 68% of total variance.

Interpretation of Principal Components

We determined correlation coefficients between initial variables and the 3 principal components (Technical Appendix Table 3). For interpretation of PCA, coefficients of interest are those whose absolute value is closer to 1.

The first principal component was negatively correlated with levels of aspartate aminotransferase and alanine aminotransferase and positively correlated with glycemia, thus differentiating children with standard levels of liver enzymes and glucose from children with signs of intoxication (higher levels of liver enzymes and lower levels of glucose). The second principal component was positively correlated with delay between disease onset and admission and leukocytes in CSF, thus differentiating children with higher levels of leukocytes (sign of infection) and less rapid progression of the disease from children with standard levels of leukocytes and a more rapid disease progression. The third principal component was negatively correlated with age, thus differentiating younger children from older children.

Three-Dimensional Representation of Patients

We obtained a projection of patients in 3-dimensional space formed by principal components (Technical Appendix Figure 4). Children with higher levels of HGA/MCPG (group 2) formed a distinct cluster in the projection space that differed from children infected with enteroviruses (group 1). Children with higher levels of HGA/MCPG had increased levels of liver enzymes and more severe hypoglycemia (negative coordinates on the first axis), more rapid progression of the disease and standard levels of leukocytes in CSF (negative coordinates on the second axis), and a younger age (positive coordinates on the third axis). Children not infected with enteroviruses for whom HGA/MCPG levels were low (group 3) or not tested (group 4) had profiles more similar to those with enteroviruses.

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Technical Appendix Table 1. Primers used for study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in children, Bac Giang Province, northern Vietnam, 2008–2011*

Primer	Gene	Sequence, 5′→3′	Reference
CoxB2/ctig74058+	3B	AGACAGGAGGAACCAGGTGAG	This study
CoxB2/ctig74058-	3B	GAAACAGTGCCTCAAGGGTGG	This study
CoxB3/ctig73505+	2B/2C	GGCTCAAGGTCAAGATCTTACC	This study
CoxB3/ctig73505-	2B/2C	GGGAAAATACTCTCTTGGCCTC	This study
CoxB5/ctig72615+	2B/2C	AGATCTTGCCAGAGGTGAAGG	This study
CoxB5/ctig72615-	2B/2C	AGTTGTTCCTGATCGCTCTGG	This study
CoxB6/ctig1095+	2A	TGCCAATGCACAATGGGGCTG	This study
CoxB6/ctig1095-	2A	CCCATAGTCACGAGTCCGATG	This study
Echo4/ctig51733+	3B/3C	AAGAGATGCTGCCCCCTTGTG	This study
Echo4/ctig51733-	3B/3C	CCTTAGCGTGGGCACTTTAGG	This study
Echo5/ctig108676+	3B/3C	TGCATACACAGGACTGCCCAA	This study
Echo5/ctig108676-	3B/3C	CTTCACTGTGCTGGAATTCCTC	This study
Echo6/ctig2864+	VP2/VP3	CGCCGAGTACAACGGATTGAG	This study
Echo6/ctig2864-	VP2/VP3	AACCAAAGACCTGCGACCCAG	This study
Echo7/ctig7637+	3D	TTCTGGGACTAGCATCTTCAAC	This study
Echo7/ctig7637-	3D	TCATAGGCATAACGGGATGAAC	This study
Echo9/ctig114121+	VP4/VP2	AGTTGCGCAGTGTTTCGCTCC	This study
Echo9/ctig114121-	VP4/VP2	GTGTTCAATGCCGCGTAGTCC	This study
Echo17/ctig26838+	2B/2C	AGTGAACGCCCAATCAGGTTAG	This study
Echo17/ctig26838-	2B/2C	CCGTATTGAGCCCGTATGTTTG	This study
Echo20/ctig90216+	3D	CACTGTTTCAAGGTCCACCAG	This study
Echo20/ctig90216-	3D	ACGCCTGGAGACAAATGACAG	This study
Echo24/ctig98651+	3B/3C	CAACACGGCCTCGTTAACTTC	This study
Echo24/ctig98651-	3B/3C	TTGTCTCCAGGCCCTAACTAC	This study
Echo25/ctig93383+	2C/3A	CTCCCTACCACCTGATCCAC	This study
Echo25/ctig93383-	2C/3A	CCTTCTCTTCTAGCGCAGCC	This study
Echo33/ctig50620+	VP1	CCTGGATCAGTCAGACGCAC	This study
Echo33/ctig50620-	VP1	ACTGGAGCATCCTGGCTCAC	This study
Echo79/ctig65990+	2B	ATGGGTGGCTAAAGAAATTTACTG	This study
Echo79/ctig65990-	2B	TTGAGCCACTCAATAAACTTCTG	This study
UnivEntero-5′ UTR-F	5′ UTR	GTACCYTTGTRCGCCTGTT	(*1*)
UnivEntero-5′ UTR-R	5′ UTR	ATTGTCACCATAAGCAGCCA	(*2*)
Ent1	5′ UTR	CGGTACCTTTGTACGCCTGT	(*3*)
Ent2	5′ UTR	ATTGTCACCATAAGCAGCCA	(*3*)
EV-040	VP1/2A	ATGTAYRTICCIMCIGGIGC	(*4*)
EV-011	VP1/2A	GCICCIGAYTGITGICCRAA	(*4*)
224	VP3	GCIATGYTIGGIACICAYRT	(*5*)
222	VP1	CICCIGGIGGIAYRWACAT	(*5*)
AN89	VP1	CCAGCACTGACAGCAGYNGARAYNGG	(*5*)
AN88	VP1	TACTGGACCACCTGGNGGNAYRWACAT	(*5*)

*Cox, coxsackievirus; ctig, contiguous; Echo, echovirus; Ent, enterovirus; EV, enterovirus; F, forward; R, reverse; UnivEntero, universal enterovirus; UTR, untranslated region; VP, virus capsid protein.

Technical Appendix Table 2. Serum concentrations of HGA and its metabolites, MCPG metabolites, and short-to-medium chain and long chain fatty acids in children with acute encephalitis syndrome, Bac Giang Province, northern Vietnam, 2010–2011*

Sample ID	Group	Hypoglycin A, nmol/L	MCPA-carnitine, nmol/L	MCPA-glycine, nmol/L	MCPF-carnitine, nmol/L	MCPF-glycine, nmol/L	Butyryl-carnitine, µmol/L	Isovalery-carnitine, µmol/L	Octanoyl-carnitine, µmol/L	Dodecanoyl-carnitine, µmol/L	Tetradecenoyl-carnitine, µmol/L	Palmitoyl-carnitine, µmol/L	Stearoyl-carnitine, µmol/L	Oleyl-carnitine, µmol/L
c1	Control	0.03	0.00	0.24	0.05	0.95	0.12	0.05	0.09	0.09	0.11	0.16	0.09	0.18
c2	Control	0.01	0.00	0.37	0.04	0.03	0.12	0.05	0.09	0.08	0.09	0.15	0.11	0.18
c3	Control	0.01	0.00	0.38	0.05	0.06	0.11	0.05	0.09	0.07	0.11	0.15	0.01	0.16
40	1	928.60	6.85	24.06	37.27	1.03	3.79	1.69	2.91	1.43	2.05	1.98	0.32	1.27
36	1	685.19	14.47	32.85	69.92	1.49	6.37	2.34	5.73	1.40	1.51	0.99	0.31	0.82
44	1	529.39	1.32	26.76	17.78	6.78	1.32	0.65	2.31	1.14	2.24	1.59	0.27	1.35
46†	1	440.14	12.97	55.64	96.31	1.83	7.60	4.63	5.06	ND	ND	ND	ND	ND
48	1	236.20	1.27	16.81	18.58	4.40	2.73	1.21	2.07	0.54	0.97	0.93	0.17	0.86
42	1	200.62	1.40	5.32	27.18	1.16	1.87	0.54	1.93	0.60	0.43	0.35	0.09	0.30
16	1	177.16	1.31	15.57	18.98	0.28	4.18	0.32	1.84	0.15	0.15	0.25	0.10	0.41
38	1	149.00	0.16	0.89	8.76	0.05	0.60	0.16	0.77	0.14	0.14	0.19	0.08	0.20
52	1	121.38	1.92	3.16	12.75	0.21	1.24	3.92	0.95	1.50	1.42	0.62	0.27	0.36
34	2	3.15	0.00	0.46	0.20	0.51	0.03	0.01	0.01	0.01	0.00	0.01	0.01	0.00
30	2	0.99	0.00	0.35	0.26	0.00	0.08	0.01	0.01	0.00	0.00	0.00	0.00	0.00
18	2	0.25	0.00	0.33	0.18	0.36	0.17	0.05	0.05	0.05	0.06	0.19	0.11	0.26
32	2	0.18	0.00	0.31	0.22	0.01	0.16	0.05	0.02	0.01	0.01	0.01	0.01	0.01
12	2	0.17	0.02	0.41	0.14	0.04	0.34	0.02	0.01	0.03	0.01	0.10	0.05	0.08
14	2	0.17	0.00	0.27	0.03	0.46	0.14	0.08	0.01	0.01	0.01	0.07	0.03	0.06
24	2	0.04	0.00	0.36	0.06	0.02	0.11	0.02	0.01	0.01	0.01	0.00	0.01	0.00
22	2	0.03	0.00	0.34	0.14	0.02	0.19	0.11	0.02	0.02	0.01	0.01	0.00	0.01
26	2	0.02	0.01	0.35	0.01	0.81	0.08	0.03	0.01	0.01	0.00	0.01	0.00	0.01
20	2	0.01	0.01	0.32	0.23	0.00	0.06	0.03	0.01	0.01	0.00	0.01	0.00	0.00
28	2	0.01	0.00	0.36	0.15	0.32	0.11	0.10	0.01	0.03	0.01	0.02	0.01	0.01
Group ratio		1,402.27	705.40	48.63	123.91	26.07	24.36	36.79	172.08	87.00	119.50	77.50	22.00	61.50

*Group ratio is the median of group 1 divided by the median of group 2. Children were divided into 2 groups: 9 had high (>100 nmol/L) values of HGA in serum (group 1), and 11 (group 2) had values below the limit of quantification (10 nmol/L), including 10 below the limit of detection (1 nmol/L) (group 2). Samples were sorted by decreasing order of HGA concentrations. Samples c1, c2, and c3 were from a healthy adult. HGA, hypoglycin A; ID, identification; MCPA, methylenecyclopropylacetyl; MCPF, methylenecyclopropylformy; MCPG, methylenecyclopropylglycine; ND, not determined.
†Ultraperformance liquid chromatography–tandem mass spectrometry was performed with 40% of the serum volume.

Technical Appendix Table 3. Characteristics of 30 hospitalized children with acute encephalitis syndrome, Bac Giang Province, northern Vietnam, 2008–2011*

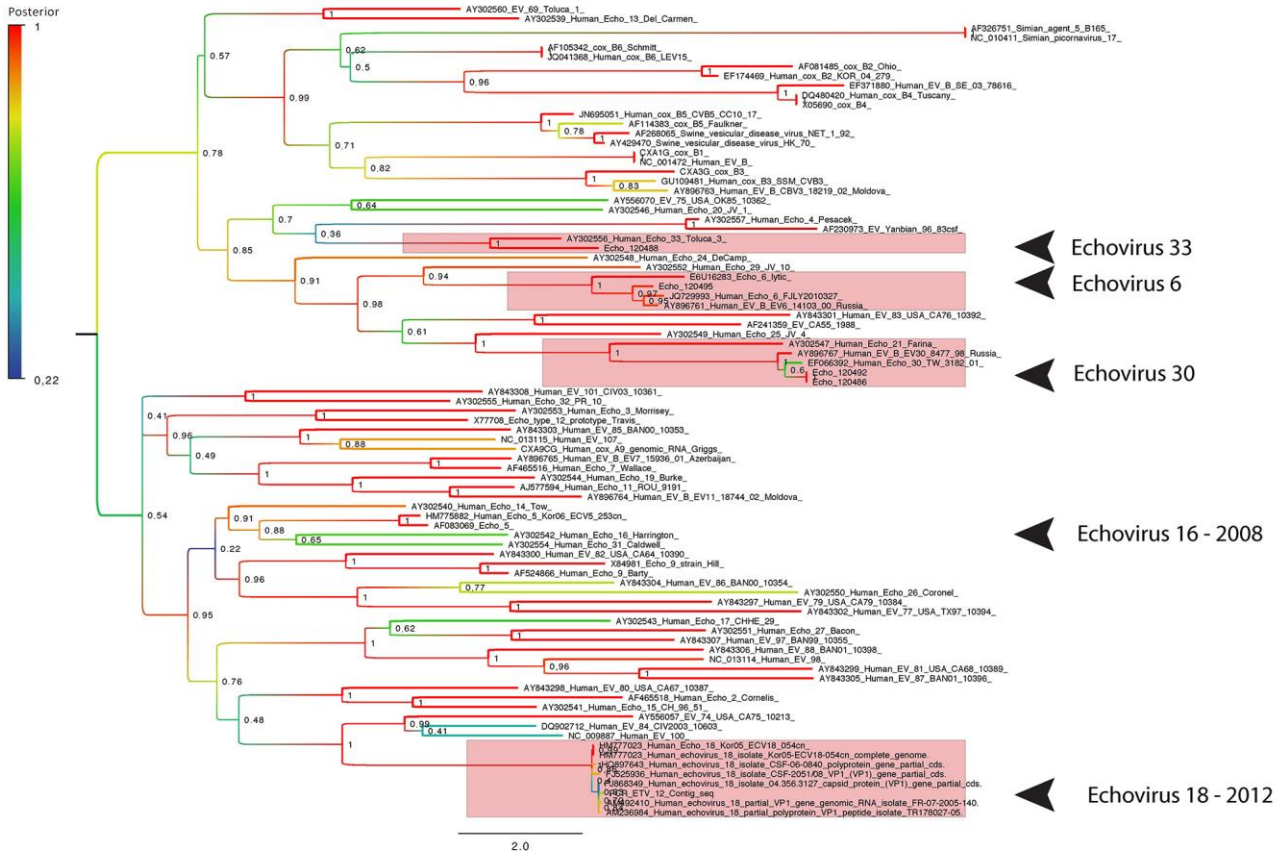
	Enterovirus negative, toxin negative (n = 7)		Enterovirus negative, toxin not tested (n = 23)	
Characteristic	No. with data	No. (%) or median (IQR)	No. with data	No. (%) or median (IQR)
Sex	7	NA	23	NA
F	NA	4 (57)	NA	12 (52)
M	NA	3 (43)	NA	11 (48)
Age, y	7	NA	23	
<2	NA	1 (14)	NA	5 (22)
2–4	NA	1 (14)	NA	6 (26)
5–9	NA	4 (57)	NA	6 (26)
10–15	NA	1 (14)	NA	6 (26)
Symptoms/signs before and at admission				
Temperature at admission, °C	7	38.0 (37.3–38.6)	22	38.0 (37.5–39.0)
Fever before admission	7	7 (100)	23	15 (65)
Headache	6	6 (100)	20	12 (60)
Seizures	7	2 (29)	23	14 (61)
Coma	3	1 (33)	17	10 (59)
Meningeal symptoms	7	7 (100)	23	19 (83)
Limb paralysis	7	1 (14)	21	4 (19)
Vomiting	6	6 (100)	18	11 (61)
Diarrhea	6	0 (0)	17	4 (24)
Days between disease onset and admission	7	2.0 (2.0–4.5)	21	3.0 (1.0–4.0)
Blood sample				
Leukocytes $\times 10^9$/L	7	16.3 (10.1–18.4)	22	17.1 (9.7–19.8)
Platelets/μL	7	226 (200–237)	19	263 (218–324)
Hemoglobin, g/L	6	62 (12–115)	11	14 (11–120)
Glucose, mmol/L	7	4.6 (3.6–6.1)	19	6.7 (4.2–8.5)
Glucose <3 mmol/L	7	1 (14)	19	1 (5)
CSF sample				
Leukocytes/mm^3	5	350 (200–350)	18	75 (16–225)
Lymphocytes/mm^3	4	45 (16–70)	11	40 (25–75)
Protein level >0.5 g/L	6	0 (0)	19	7 (37)
Transparent appearance of CSF	5	4 (80)	18	18 (100)
Liver enzymes at or after admission				
ALT, IU/L	7	31 (20–42)	14	21 (14–28)
AST, IU/L	7	43 (38–53)	14	38 (26–63)

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CSF, cerebrospinal fluid; IQR, interquartile range; NA, not applicable..

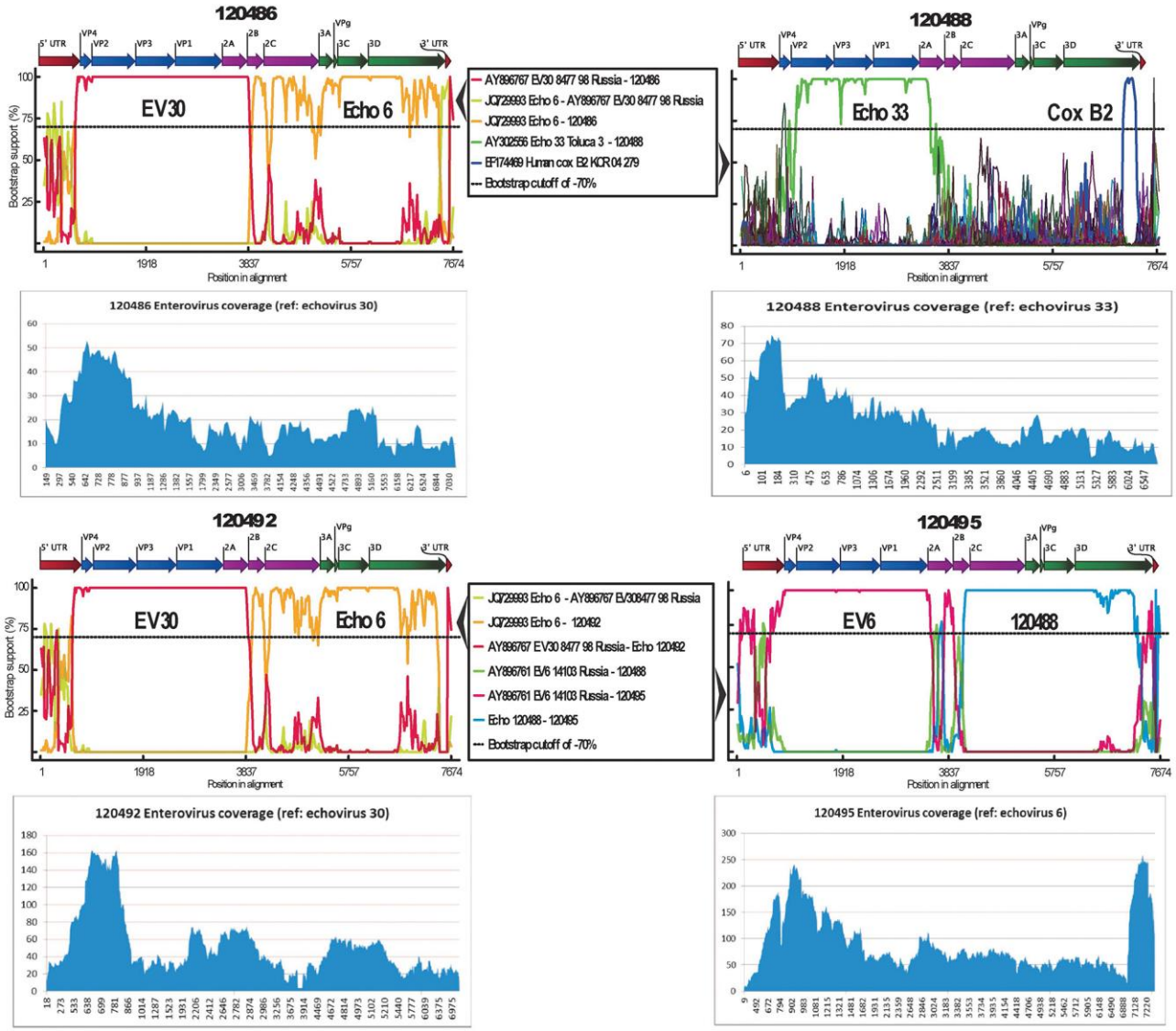
Technical Appendix Table 4. Correlation coefficients between initial variables and 3 principal components in study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in hospitalized children, Bac Giang Province, northern Vietnam, 2008–2011*

Variable	PC1	PC2	PC3
Age	−0.317	0.297	−0.768
Delay between disease onset and admission	0.290	0.803	0.015
Glucose level	0.528	0.130	0.409
Leukocytes in CSF	−0.096	0.803	0.118
ALT level	−0.794	−0.006	0.184
AST level	−0.771	0.175	0.398

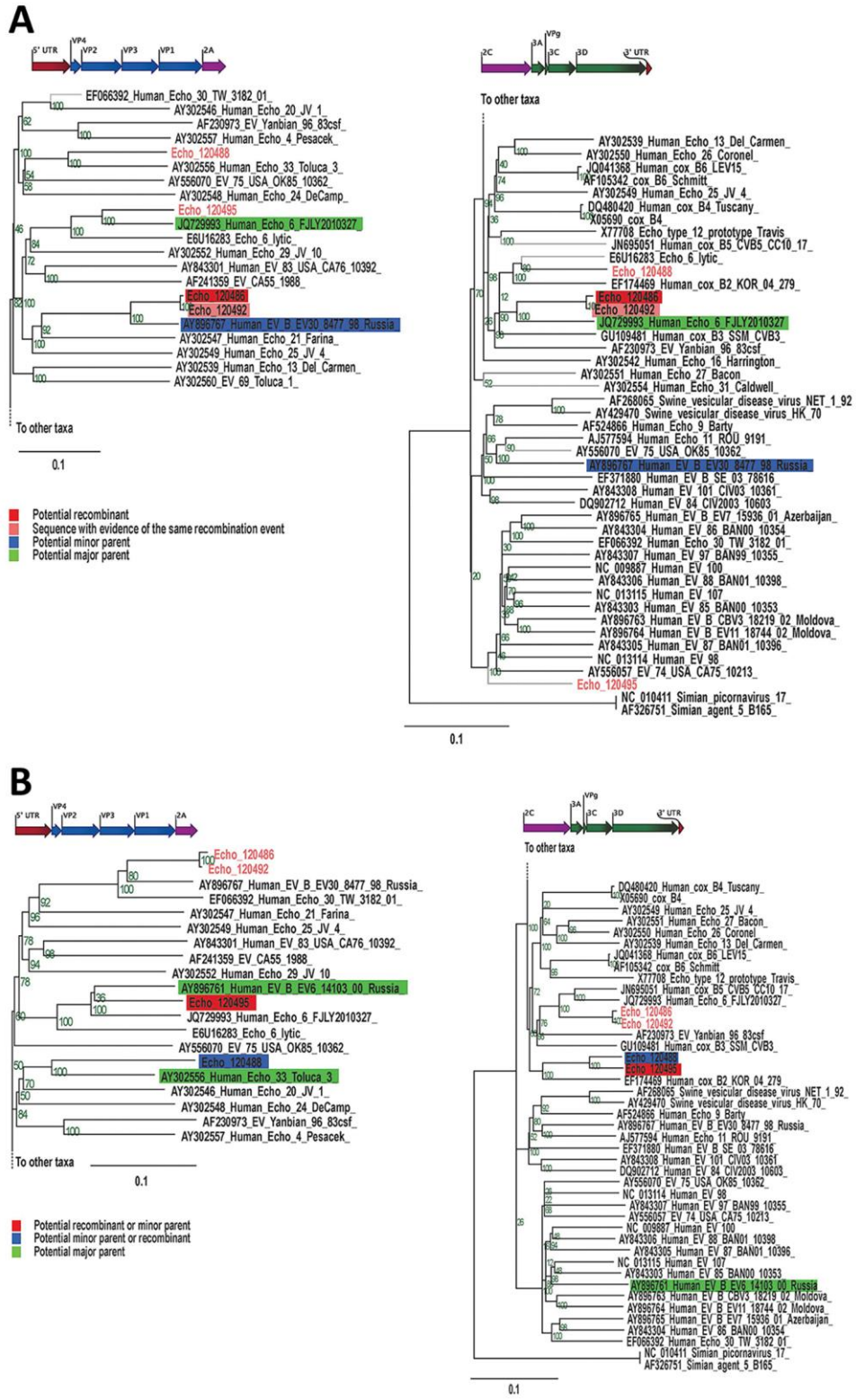
*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CSF, cerebrospinal PC, principal component.



Technical Appendix Figure 1. Virus capsid protein 1–based phylogeny of enteroviruses, showing clustering of strains detected in this study with representatives of human enterovirus B serotypes. Numbers along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

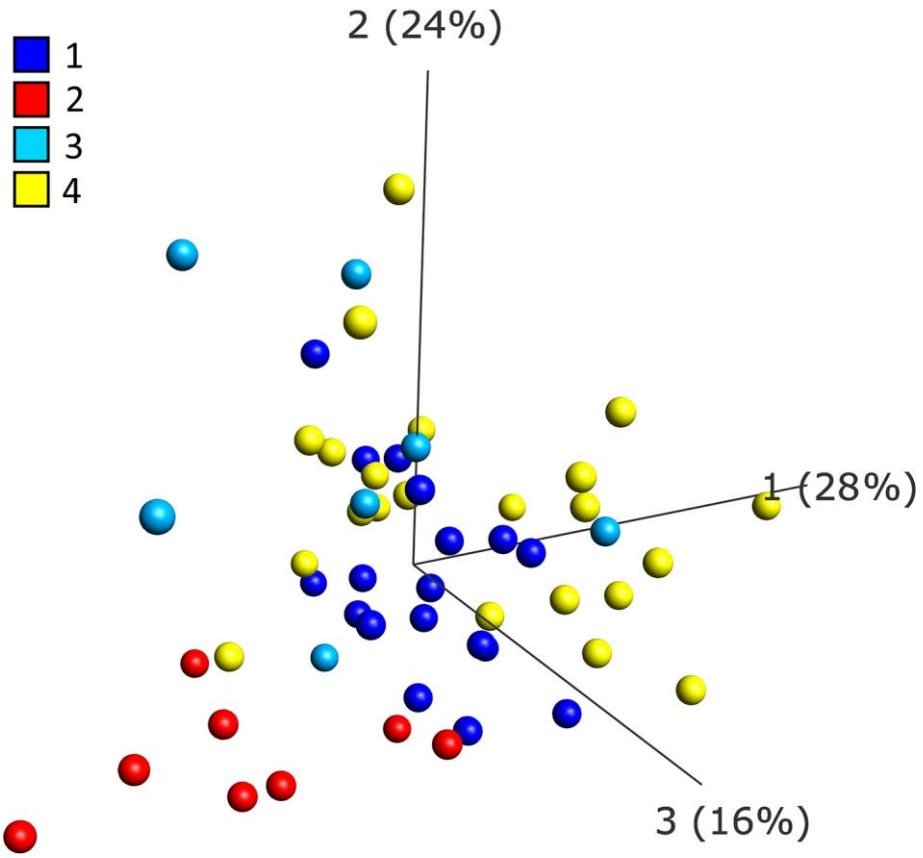


Technical Appendix Figure 2. Putative recombination patterns of enteroviruses 120486, 120488, 120492, and 120495 with other human enterovirus B strains and next-generation sequencing coverage of genomic sequences obtained from cerebrospinal fluid samples. For each enterovirus strain genomic sequence, the coverage is represented spanning genome, and the corresponding open reading frame map is depicted. Diagrams illustrate the association likelihood between sequences putatively involved in recombination events with the 4 genomes presented. Putative breakpoints were detected and parental sequences were identified on the basis of a combination of 5 algorithms (RDP, SiScan, BootScan, MaxChi, and Chimaera) applied to a matrix containing representative human enterovirus B genomes with a sliding window of 200 nt incremented every 20 nt. Echo, echovirus; EV, enterovirus; Cox, coxsackievirus; ref, referent; UTR, untranslated region. VP, virus capsid protein.



Technical Appendix Figure 3. Phylogenetic analysis of putative recombination patterns of enteroviruses detected. A) Phylogenies of beginning and end regions of genomes of human enterovirus B strains 12486 and 120492. B) Phylogenies of beginning and end regions of genomes of human enterovirus B strains 12488 and 120495. Clear incongruences emerge from phylogenetic reconstructions according to 3′ versus 5′ regions of the

genome, given the similarity of strains 120486 and 120492 and the proximity of strain 120486 with enterovirus 30 and the proximity of strain 120492 with echovirus 6 for VP (5′) and 2C to 3D (3′) genes. Similarities were observed between strains 120488 and 120495 in their 3′ genome regions, which contrasted with strong similarity of the VP (5′) gene of strain 120488 with enterovirus 33 and the VP (5′) gene of strain 120495 with enterovrius 6. Numbers along branches are bootstrap values. Scale bars indicate nucleotide substitutions per site. Cox, coxsackievirus; Echo, echovirus; EV, enterovirus; UTR, untranslated region. VP, virus capsid protein.



Technical Appendix Figure 4. Projection of patients in the 3-dimensional space formed by principal components. Colors indicate the 4 groups of patients described in our study. 1, patients positive for enteroviruses (n = 19) (dark blue); 2, patients positive for toxins (n = 8) (red); 3, patients negative for enterovirus and toxins (n = 7) (light blue); 4, patients negative for enterovirus and not tested for toxins (n = 23) (yellow).